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A RAPID AND SENSITIVE METHOD FOR THE QUANTITATION OF LEGIONELLA--ETC(U)
NOV 80 J A MANGIAFICO, K W HEDLUND, A R KNOTT

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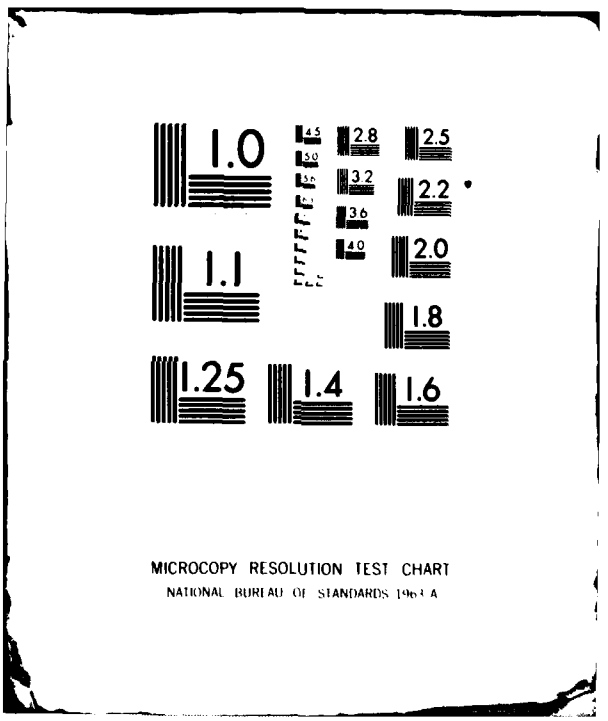
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ABSTRACT

A reversed passive hemagglutination test was developed to assay concentrations of soluble antigen of Legionnaires disease (Legionella pneumophila) in human urine samples. The test is highly sensitive, being able to detect as little as 0.0002 μg of antigen. Preliminary results with this test on serial urine and serum samples from a patient with legionellosis show that measurable amounts of antigen are present in urine during the course of the illness. However, no antigen could be detected in the patient's serum.

The reversed passive hemagglutination test (RPHA) has been successfully used in the past for detection and or assay of tetanus toxin (2) and staphylococcal enterotoxin B in culture filtrates and in food samples (5).

The test is rapid, reliable, highly sensitive and does not require extensive or exotic reagents. With the reported success of detecting Legionnaires' disease (LD) antigen in sputum and urine samples (1, 4), we believed that a RPHA test might be an easy and rapid method for accomplishing this aim. We describe in this report the development of a RPHA test for the detection and quantitation of soluble LD antigen. The report also includes results obtained (with this method) from serial urine and serum samples obtained from a LD patient.

MATERIALS AND METHODS

Erythrocyte preparation. Sheep red blood cells (SRBC) were collected in equal volume of Alsever's solution and stored at 4°C for at least 3 days prior to use. The SRBC were washed three times with 0.9% NaCl and resuspended to 2.5% in phosphate buffered saline (PBS), pH 7.2. The cells were tanned by mixing equal volumes of 2.5% SRBC and 1:20,000 tannic acid in PBS pH 7.2 and incubating the mixture in 37°C water bath for 15 min. The tanned cells were then washed three times with PBS, pH 7.2, and resuspended to 2.5% in PBS, pH 6.4. Tanned cells were sensitized by mixing with an equal volume of optimally diluted goat anti-LD globulin; the mixture was incubated for 15 min at 37°C. Sensitized cells were washed twice with PBS, pH 7.2, with 1% normal rabbit serum (PBS-NRS) and resuspended to 0.7% in PBS-NRS for use in the microtiter test system.

Soluble antigen preparation. Cultures of the Washington strain of Legionella pneumophila on modified Mueller-Hinton medium (3) were originally obtained from Drs. McDade and Shepard (Center for Disease Control, Atlanta, Ga). Soluble antigen was prepared from organisms grown on Mueller-Hinton medium as previously described (4). The purified final product was lyophilyzed and stored at -20°C.

Globulin preparation. Anti-LD globulin was prepared from goats hyperimmunized with the Washington strain of L. pneumophila. Goat serum was saturated with $(NH_4)_2SO_4$ to 50%. The precipitate was washed several times with saturated $(NH_4)_2SO_4$, dialyzed against 0.9% NaCl and stored at -20°C. This globulin preparation was found to contain 820 µg/ml of antibody nitrogen by the Kjeldahl test. Normal goat IgG was obtained from Chappel Laboratories, Inc., Cochranville, Pa. In other studies not described here, rabbit anti-LD globulin gave less satisfactory results than the goat anti-LD globulin. The concentration of globulin

required for optimal sensitization of tanned SRBC was determined by titrating known quantities of standard soluble antigen using tanned SRBC with varying concentrations of globulin. Based on these box titrations, goat anti-LD globulin containing 80 µg/ml of antibody nitrogen was used in the sensitization procedure.

Test samples were initially diluted 1:8 in PBS-NRS, heat-inactivated for 30 min at 60°C and absorbed with 0.1 ml of packed SRBC. Subsequent serial two-fold dilutions were prepared in "Titertek" U-plates (Linbro Scientific, Hamden, Conn.) using 0.05-ml microtiter loops and PBS-NRS diluent. To each dilution was added 0.025 ml of 0.7% tanned, sensitized SRBC. Plates were incubated at room temperature for 2-3 h and read for agglutination patterns.

Included in each run was an antigen standard with a known starting concentration. Antigen concentrations in samples were calculated by multiplying the reciprocal of the greatest dilution that reacted for each sample by the smallest amount of standard antigen that gave a positive result.

In order to rule out nonspecific agglutination reactions, samples were tested against tanned, nonsensitized SRBC and tanned SRBC sensitized with normal goat IgG. Other controls included in each run were a normal negative, tanned cells plus diluent, normal SRBC plus diluent, and sensitized cells plus diluent.

RESULTS

The RPHA test was initially tested for its capability of detecting and measuring LD antigen prepared from the standard Washington strain soluble antigen. With the globulin used in these studies, 0.0012 µg/ml of LD antigen was generally the smallest amount that caused a distinct hemagglutination (HA) positive pattern. The mean end-point for about 12 RPHA tests, run against the standard Washington strain soluble antigen was 0.0043 µg/ml (0.0002 µg in the 0.05-ml volume used in the test).

Frozen serial urine and serum samples were obtained from a patient hospitalized elsewhere with clinically diagnosed LD. This infection was subsequently confirmed serologically as LD, serogroup 1, by microagglutination and indirect fluorescent antibody tests. Urine and serum samples from this patient were tested by the RPHA test to evaluate its capability to detect and quantitate soluble LD antigen present in the specimens. Results obtained by the RPHA test are shown in Table 1. Antigen was detected in measurable quantities in all of the urine samples tested, with the peak concentration found in the June 20, 1980, urine sample, 4 days after the patient was admitted to the hospital. A second, smaller peak of antigen was detected in the July 7 urine sample, 4 days after the patient was scheduled to terminate antibiotics treatment. However, occurrence of the second antigen peak (7 July) could not be attributed to any particular circumstance, since precise information on the times and methods used to collect the urine specimens was not available. Measurable quantities of LD antigen were detected in the urine for at least 30 days after it had reached its peak.

Although attempted on several different occasions, LD antigen could not be detected in any of the serum samples (acute or convalescent) obtained from this patient.

DISCUSSION

This study shows that the RPHA can be used to detect and quantitate soluble antigen of LD. The test is rapid requiring only 2 h before it can be read. Samples can be prepared, tested and read easily within an 8-h work day.

The test is also sensitive, capable of detecting 0.0012 µg/ml or 0.0002 µg of antigen with the volume used in the test. This sensitivity is also an advantage in quantitating small concentration of antigens in natural fluids, since the samples to be tested do not require prior concentration.

The test as described here is fairly simple to run. It does not require exotic reagents or highly purified or fractionated antibody globulins. The rapidity, sensitivity and simplicity of the RPHA test make it well suited as a research tool as well as for diagnostic serology.

Although the human urine and serum samples are from only one patient, the results substantiate the findings of Tilton (6) and of Berdal et al. (1) that LD antigen is excreted in the urine of infected individuals. Our findings suggest that antigen maybe excreted in the urine starting early in the infection. The amounts excreted fluctuated but were subject to quantitation for at least 30 days after they had reached their peak in the urine. The inability to detect any antigen from this patient's serum samples is unexplainable at this time. Part of the time it is conceivable that the antigen is masked by antibody. It may also suggest that antigen is effectively and rapidly filtered from the patient's serum and concentrated in the urine.

ACKNOWLEDGMENTS

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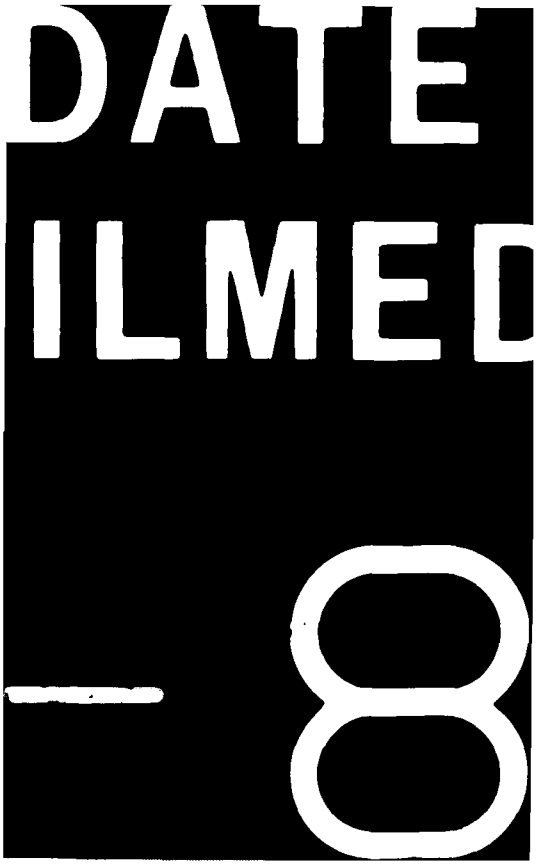
TABLE 1. L. pneumophila antigen measured in the urine and serum from a patient with LD by RPHA test

Date of sample (1980)	Amount of antigen detected (μg/ml)	
	Urine	Serum
June 17	≤ 0.128	< 0.004
June 18	0.128	0.004
June 20	16.4	NS
June 23	NS[a]	< 0.004
June 26	1.024	NS
June 30	0.512	< 0.004
July 7	4.096	0.004
July 15	2.048	0.004
July 19	NS	0.004
July 22	1.024	0.004

[a]NS denotes no sample was taken for that day.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